Viruses **2014**, *6*, 4609-4627; doi:10.3390/v6114609

OPEN ACCESS

viruses

ISSN 1999-4915

www.mdpi.com/journal/viruses

Review

Implication of Human Endogenous Retrovirus Envelope Proteins in Placental Functions

Adjimon Gatien Lokossou, Caroline Toudic and Benoit Barbeau *

Département des Sciences Biologiques and Centre de recherche BioMed,
Université du Québec à Montréal, 2080 Saint-Urbain, Montréal, PQ H2X 3X8, Canada;
E-Mails: gatien.lokossou@gmail.com (A.G.L.); caroline.toudic22@gmail.com (C.T.)

* Author to whom correspondence should be addressed; E-Mail: barbeau.benoit@uqam.ca;
Tel.: +1-415-985-3000 (ext. 4576); Fax: +1-541-987-4647.

External Editor: Welkin Johnson

Received: 11 October 2014; in revised form: 2 November 2014 / Accepted: 7 November 2014 / Published: 24 November 2014

Abstract: Human endogenous retroviruses (ERVs) represent 8% of the total human genome. Although the majority of these ancient proviral sequences have only retained non-coding long terminal repeats (LTRs), a number of "endogenized" retroviral genes encode functional proteins. Previous studies have underlined the implication of these ERV-derived proteins in the development and the function of the placenta. In this review, we summarize recent findings showing that two ERV genes, termed Syncytin-1 and Syncytin-2, which encode former envelope (Env) proteins, trigger fusion events between villous cytotrophoblasts and the peripheral multinucleated syncytiotrophoblast layer. Such fusion events maintain the stability of this latter cell structure, which plays an important role in fetal development by the active secretion of various soluble factors, gas exchange and regulation of fetomaternal immunotolerance. We also highlight new studies showing that these ERV proteins, in addition to their localization at the cell surface of cytotrophoblasts, are also incorporated on the surface of various extracellular microvesicles, including exosomes. Such exosome-associated proteins could be involved in the various functions attributed to these vesicles and could provide a form of tropism. Additionally, through their immunosuppressive domains, these ERV proteins could also contribute to fetomaternal immunotolerance in a local and more distal manner. These various aspects of the implication of Syncytin-1 and -2 in placental function are also addressed in the context of the placenta-related disorder, preeclampsia.

Keywords: human endogenous retrovirus; Syncytin-1 and -2; placenta; syncytiotrophoblast; immunotolerance; exosomes; pre-eclampsia

1. Introduction

It is now well established that viral relics, named endogenous retroviruses, derived from ancestral infectious retroviruses, have made an important entry in vertebrates through their germ cells [1,2]. The similarities of their genomic structure (consisting of *gag*, *pro*, *pol* and *env* genes flanked by two long terminal repeats (LTRs)) with known retroviruses were thus an essential clue for their initial identification. Furthermore, upon infection and integration in germ cells, resulting proviral DNAs likely further expanded their copy number by reinfection of germ cells [1]. Through evolution, the loss of a functional Env protein by mutation (insertions, deletions, substitutions) and/or epigenetic modifications of the locus have potentially rendered these retroviral sequences unable to produce infectious virions, although retrotransposition might have led to the further increase in their copy number [1,3].

Although the exact nature and function of these sequences remain largely unknown, recent studies on ERVs in humans (formerly termed HERVs) have provided intriguing mechanisms of action for some of the encoding genes. ERV sequences represent up to 8% of our genome and are largely composed of solo LTRs (90%), resulting from recombination events between these flanking elements. These ERVs are classified into different families, which have been reordered in three groups based on homology [4–6]. Families of ERVs are normally identified with a letter, corresponding to the specific amino acid anchored to the tRNA required for the initial first strand DNA synthesis in the retrotranscription step of retroviral replication (for example, the identification of ERVW-1 is based on the tryptophan (W) amino acid attached to the tRNA, which was formally needed for retrotranscription of its genomic RNA). Interestingly, sequence analysis of several ERV sequences also revealed that typical retroviral ORFs are still present in certain loci, although they have acquired several mutations during evolution. In fact, no ERVs, including the most recently acquired ERV-K members, have been shown to be replication competent [7–9]. Despite the fact that ERVs have been linked to various diseases, such as multiple sclerosis, cancer and diabetes [10–13], the retention of their genes during evolution suggests that they have provided a beneficial role to human survival. In this respect, studies that have highlighted a strong implication of ERV genes in the development and the function of the placenta represent the best example of their beneficial nature. This review will focus on some of the recent findings on the association between ERVs and this important organ.

2. ERVs and Placenta Development

A number of studies have highlighted the implication of various ERV genes in normal placenta development. The human placenta, an indispensable organ for intrauterine fetal growth, is composed of various cell types. These cells include extravillous and villous cytotrophoblasts, the latter capable of further differentiation into an overlaying structure, known as the syncytiotrophoblast. This cell layer is a multinucleated cellular barrier, which is in direct contact with maternal blood. The syncytiotrophoblast plays a fundamental role through the optimization of the proper exchange of

nutrients and hormones between the mother and the fetus and through the production of important soluble factors, such as human chorionic gonadotropin (hCG) and human placental lactogen (hPL) [14–16]. Additionally, the syncytiotrophoblast maintains fetomaternal tolerance by continuous interaction and monitoring of surrounding dendritic cells, macrophages, T-lymphocytes and natural killer cells. This constant monitoring allows a firm regulation of the immunosuppressive state that is absolutely required to prevent fetal rejection [17–19]. The placenta, and particularly, residing cytotrophoblasts, actively expresses a number of ERV envelope (Env) genes [20–24]. Based on these previous findings, a set of pivotal studies have demonstrated that two Env proteins, termed Syncytin-1 and Syncytin-2, were likely inducing fusion between underlying villous cytotrophoblasts and the syncytiotrophoblast layer, thereby contributing to the constant renewal and stability of this highly dynamic structure [20,22,25–27]. Syncytin-1 and Syncytin-2 proteins are encoded by two different ERV loci, *i.e.*, ERVW-1 and ERVFRD-1, which are located on chromosome 7 and 6, respectively. Although both proteins are expressed in the placenta, certain differences exist regarding their localization and expression patterns. In fact, expression of Syncytin-1 is mostly limited to villous and extravillous cytotrophoblasts [22,28–30]. A number of studies have thereby addressed the mechanism of the regulation of Syncytin-1 expression and have led to the characterization of its promoter, which is partly embedded in the 5' LTR and is dependent on the transcription factor GCM1 along with others, such as Sp1 and GATA family members [31–35]. In addition, DNA methylation and histone H3K9 trimethylation at the Syncytin-1 locus have been reported to be important epigenetic modifications that silence ERVW-1 expression [36–39]. Another report has further indicated that splicing could be an alternative mechanism of the regulation of Syncytin-1 expression [40]. Similarly to other retroviral envelope proteins, Syncytin-1 interacts with receptors to mediate fusion. Indeed, conclusive reports have identified two sodium-dependent neutral amino acid transporters, namely solute carrier family 1 members 4 and 5 (SLC1A4 and SLC1A5) (otherwise known as ASCT1 and ASCT2), as its receptors [41–43].

However, a certain controversy has been underscored as to the role of Syncytin-1 in trophoblast fusion and its cellular distribution, which shows variation in terms of its localization in different trophoblast cell populations [44]. The discovery of Syncytin-2 has been an important finding, which has further shed light on the complexity of trophoblast fusion [20,45]. Our team has, in fact, demonstrated that Syncytin-2 played a more decisive role in cytotrophoblast fusion, when compared to Syncytin-1 [23]. Indeed, transfection experiments of siRNA in the human BeWo cell line and in primary cytotrophoblasts showed a more pronounced decrease in fusion events upon repression of Syncytin-2 expression in comparison to the conditions in which Syncytin-1 was silenced. Syncytin-2 expression is GCM-1-dependent and is specifically expressed in villous cytotrophoblasts, although we have shown an increase of its expression upon differentiation of primary cytotrophoblasts in cell culture [23,45,46]. Syncytin-2 has been associated with a single receptor, the major facilitator superfamily domain containing 2a (MFSD2a), which belongs to the carbohydrate carrier family and has recently been associated with the transport of the essential fatty acid, docosahexaenoic acid [47,48]. MFSD2a is forskolin-inducible, is expressed in the syncytiotrophoblast and has been confirmed to be implicated in fusion [48,49]. In addition to Syncytin-1 and -2, a subsequent study by Blaise *et al.* also showed that two other ERV envelope proteins, EnvV and EnvP(b), are also expressed in the placenta [21]. Interestingly, the EnvP(b) protein has retained its fusogenic potential, although

being less specifically expressed in the placenta. However, using the trophoblast-like BeWo cell line model, results from our team did not support a role for this Env protein in trophoblast fusion [50]. As for EnvV, two loci have been identified, namely ERVV-1 and ERVV-2 [51], resulting from gene conversion. Recent reports suggest that one of these loci has lost its fusogenic activity in humans, but is fusogenic in placenta of Old World monkeys [52]. From these various results, it should also be underscored that in addition to the *envV* gene potentially associated with placentation in Old World monkeys, a number of *env* genes designated as Syncytin or Syncytin-like, have been strongly suggested to bear a specific role for normal placenta development in a wide variety of mammalian representatives, such as mouse, ruminants, sheep, dogs and cats [53–63]. This is a clear demonstration of convergent evolution and greatly contributes to our understanding of the importance of ERVs in mammalian evolution.

Syncytin proteins are typical retroviral-like envelope proteins and have retained the general structure of these glycoproteins (Figure 1) [64]. Even though they originate from exogenous retroviruses having infected primate ancestors over several million years ago, these proteins have remarkably conserved their fusogenic potential. As depicted in Figure 1, both Syncytin-1 and -2 are synthesized as polyproteins, which are cleaved into surface (SU) and transmembrane (TM) subunits by the cellular furin protease [65,66].

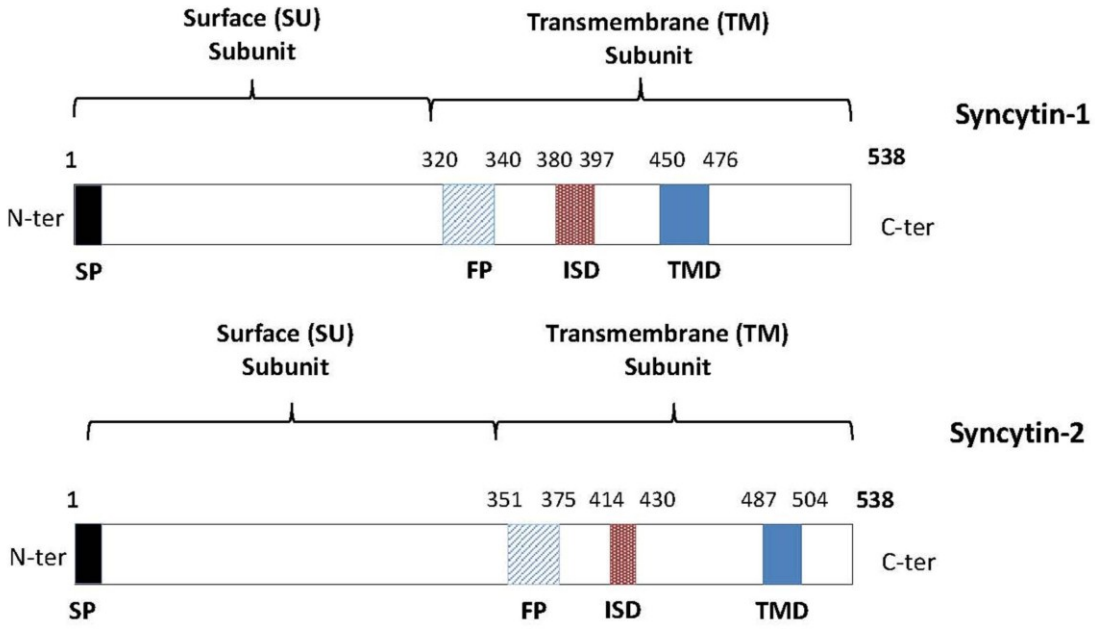

Figure 1. Schematic presentation of the functional domains of Syncytin-1 and -2. Similarly to other retroviral glycoproteins, Syncytin-1 and Syncytin-2 are synthesized as inactive precursors, which are then cleaved into two functional subunits: the SU and the TM subunit. SU is responsible for receptor binding and TM mediates the fusion. Both proteins are 538 amino acids long and harbor a fusion peptide (FP), a functional immunosuppressive domain (ISD) and a transmembrane domain (TMD) in their TM subunit. As a membrane protein, the polyprotein also possesses a cleaved signal peptide (SP) at its amino end.

The SU component of the envelope protein is required for receptor recognition, and the receptor-binding domain has been mapped to the NH_2 end of Syncytin-1 [67]. The TM subunit anchors the whole envelope glycoprotein complex to the membrane through its transmembrane domain (TMD) and is directly responsible for membrane fusion between target cells and Syncytin-expressing cells upon insertion of its fusion peptide (FP) to the plasma membrane. Further studies have also revealed the functional resemblance of Syncytin-1 and -2 in comparison to common exogenous retroviruses, with a need to form trimers for proper fusion [66,68]. As an additional interesting feature of Syncytin proteins, again shared with a certain number of exogenous retroviruses, the presence of an immunosuppressive domain (ISD) has also been demonstrated in these ERV proteins [69].

3. Syncytin-1 and Syncytin-2: Potential Mediators of Immune Tolerance

Due to the presence of their ISD domain, early identification of both Syncytin-1 and-2 suggested that these proteins could be involved in the fetomaternal tolerance state prevailing during pregnancy. It is indeed known that exogenous retroviruses frequently induce severe immunosuppression in both human and animals [70]. Although the exact molecular mechanisms and interacting partners involved in the modulation of the immune response are not known, retroviral envelope proteins are likely mediators of this immune dysfunction, which depends on the presence of the immunosuppressive domain, as recently illustrated by Schlecht-Louf *et al.* with Friend murine leukemia virus (F-MLV) and a feline leukemia virus (FeLV) vaccine [71,72]. *In vitro* and *in vivo* studies have in fact shown that a synthetic retroviral 17 amino acid peptide representing the ISD is extremely immunosuppressive [69,73–75]. Moreover, contrary to a previous report [76], we showed that the endogenous retroviral envelope Syncytin-1 inhibits LPS/PHA-stimulated cytokine responses in human blood cells. This report suggested that Syncytin-1 is immunosuppressive and may equally be relevant to maternal immunotolerance [77]. More studies are needed to better appreciate the role of Syncytin-1 and -2 in the regulation of the immune response in the vicinity of the fetus. However, recent findings showing that these proteins are associated with various types of extracellular microvesicles are now helping to provide more adapted mechanisms of action of these proteins toward immunotolerance [77–79].

4. Exosomes and the Placenta

The placenta releases extracellular microvesicles of different types, which include exosomes and syncytiotrophoblast microparticles [80–83]. Research on exosomes, most notably in the field of placenta research, has been increasing in importance over recent years and has demonstrated that these vesicles are involved in many different normal and pathological processes. Exosome-associated proteins mediate different exosomal functions, such as intercellular communication, induced cell signaling and miRNA-dependent modulation of gene expression. Recent findings suggest that incorporated ERV Env proteins are also playing an active role [77,84].

Exosomes are part of a growing list of cellular microvesicles, including microvesicles shedding from the plasma membrane and apoptotic blebs. Originally described in rat and sheep reticulocytes [85–88], they were first functionally associated with the disposal of unnecessary proteins. It is now well established that the biological function of such microvesicles is oriented towards cell-to-cell communication. Exosomes are released by a large range of cells, including immune

cells [89–91], neural cells [92,93], stem cells [94,95], placenta cells [77,84,96,97] and many cancer cells [98], and can be isolated from different body fluids, such as serum, urine, cerebrospinal fluid and amniotic fluid (reviewed in [99]). Several characteristics allow the distinction of exosomes from the other cellular microvesicles. Firstly, exosomes are microvesicles (40 nm–100 nm) that follow the endocytic pathway instead of directly budding from the plasma membrane. Secondly, they have an homogenous cup-shaped structure, when observed by electron microscopy, Finally, their buoyant density ranges between 1.13 g/mL and 1.19 g/mL on a sucrose gradient [100]. Exosomes are generated as intraluminal vesicles (ILVs) contained in a subtype of late endosomes called multivesicular bodies (MVBs). The general process leading to the formation of exosomes can be summarized in three major steps. Firstly, the inward budding of the membrane of late endosomes leads to the formation of ILVs within MVBs. Secondly, the newly generated MVBs can either fuse with lysosomes, thus leading to the degradation of their content, or be directed to the plasma membrane [101]. Thirdly, the MVB membrane fuses with the plasma membrane, thus allowing exosome secretion. Although the protein composition of exosomes varies according to their originating cell, proteome analyses have highlighted the presence of constitutive proteins that belong to late endosome/MVB compartments. Indeed, the tetraspanins, CD9, CD63, CD81, and the ESCRT-related proteins, Alix and TSG101, are constituents of nearly all exosomes and are markers used for the detection of exosomes [91,99,102]. The acetylcholine esterase (AChE) activity has also been associated with exosomes in several studies and is a useful tool to control for exosome isolation and purification [84,88,103,104].

Exosome functions depend on their protein and RNA content and operate through direct contact with surface proteins of target cells or through modification of cell signaling or cellular gene expression upon fusion. This is particularly relevant, as exosome-associated miRNAs have the potential to alter the gene expression of targeted cells and thereby impact cell fate. In fact, many mRNA and miRNA have been isolated from different cell- or body fluid-derived exosomes, and a database is now available that combines all of the data currently available on the protein/RNA/lipid composition of exosomes (http://www.exocarta.org) [105]. More specifically, in relation to the placenta, a number of reports have focused on the miRNA and protein content of placenta-derived exosomes and have shown a very complex, yet partly placenta-selective, miRNomic profile [97]. Recent findings have further revealed that the placenta-specific miRNA cluster, C19MC, was a component of primary trophoblast-derived exosomes [106]. These latter miRNAs are likely to contribute to exosome function and, in fact, have recently been suggested to limit viral infection on recipient cells in an autophagy-dependent manner [107,108]. Exosomes originating from different placental cell types have also been reported to act on endothelial cell and smooth muscle cell migration, although the precise mechanism remains to be determined [109–111]. Despite these interesting findings, no clear clues as to how these exosomes are able to deliver their content to cell targets are currently available. Our team has recently suggested a mechanism of delivery, whereby ERV Env proteins incorporated at the surface of cytotrophoblast-derived exosomes bind to their specific receptors [84]. In fact, an association between viruses and exosomes has been previously described for many viral families, including ERVs [112]. In our study, we have demonstrated that exosomes isolated from the culture supernatant of primary villous cytotrophoblasts or from the serum of pregnant women harbored both Syncytin-1 and -2 (Figure 2).

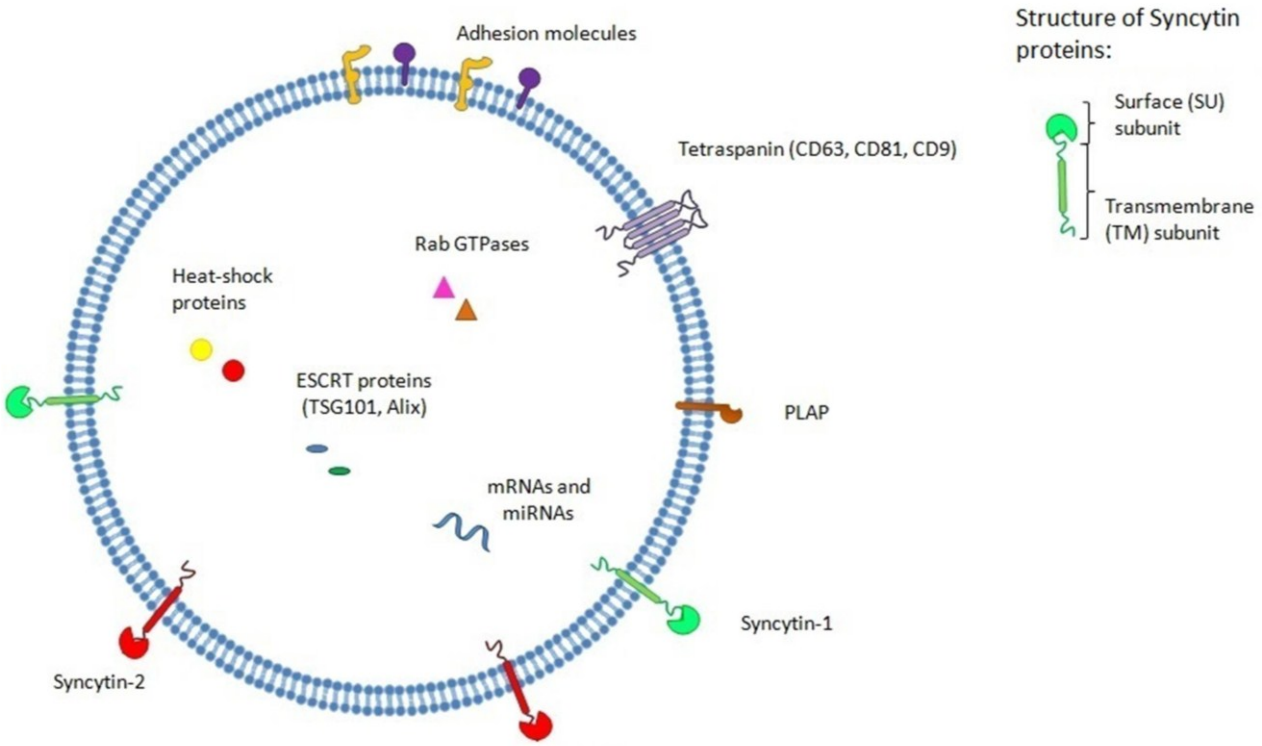

Figure 2. Syncytin-1 and -2 are present at the surface of placental exosomes. Schematic representation of a human placental exosome harboring Syncytin proteins on its surface. Both incorporated Syncytin-1 and -2 are composed of SU and TM subunits. The figure also depicts a certain number of proteins and RNA species most commonly associated with placental exosomes.

The incorporation of Syncytin-1 and -2 at the surface of placental exosomes is also in line with our previous findings showing the important intracellular distribution of Syncytin-1 and Syncytin-2 in addition to their expected plasma membrane localization in both stimulated BeWo cells and primary cytotrophoblasts [23]. In our report on exosomes, our results further indicated that depletion of either Syncytin-1 or -2 reduced the uptake of resulting exosomes by BeWo cells. These data thus suggest that both Syncytin-1 and -2 are involved in the uptake of placental exosomes by target cells after binding to their respective receptor followed by entry through the endocytic pathway. Several adhesion proteins and ligands, such as integrins, annexins, Claudin-1 and ICAM-1, are found at the surface of exosomes and most likely contribute to the binding of exosomes to the cell surface. However, the delivery of exosome content implies the fusion of the exosome membrane with the endocytic vesicle, and in this perspective, Syncytin-1 and -2 might make a crucial contribution toward this process. Thus, we are proposing a model by which, through incorporating Syncytin proteins, placental exosomes first contact specific (SCL1A4/5 and/or MFSD2a) receptors with potential co-receptor binding mediated by other cell surface complexes (Figure 3). Exosomes are then internalized in target cells through a clathrin-coated endocytic process. The exosome-associated Syncytin-1 and -2 bound to their respective receptors could then mediate fusion with the endosomal membrane, thereby specifically delivering their content to these cells. This Env protein-dependent interaction between exosomes and target cells could thus refer to a form of exosome tropism, which is comparable to the combination of specific

receptors and co-receptors that allows the infection of specific target cells by viruses. Although more experiments are needed to discern the exact mechanism underlying the functional role of these ERV Env proteins in the intracellular trafficking of internalized placental exosomes, it is clear that they potentially provide a selective interaction with target cells, which could include endothelial cells and villous/extravillous cytotrophoblasts.

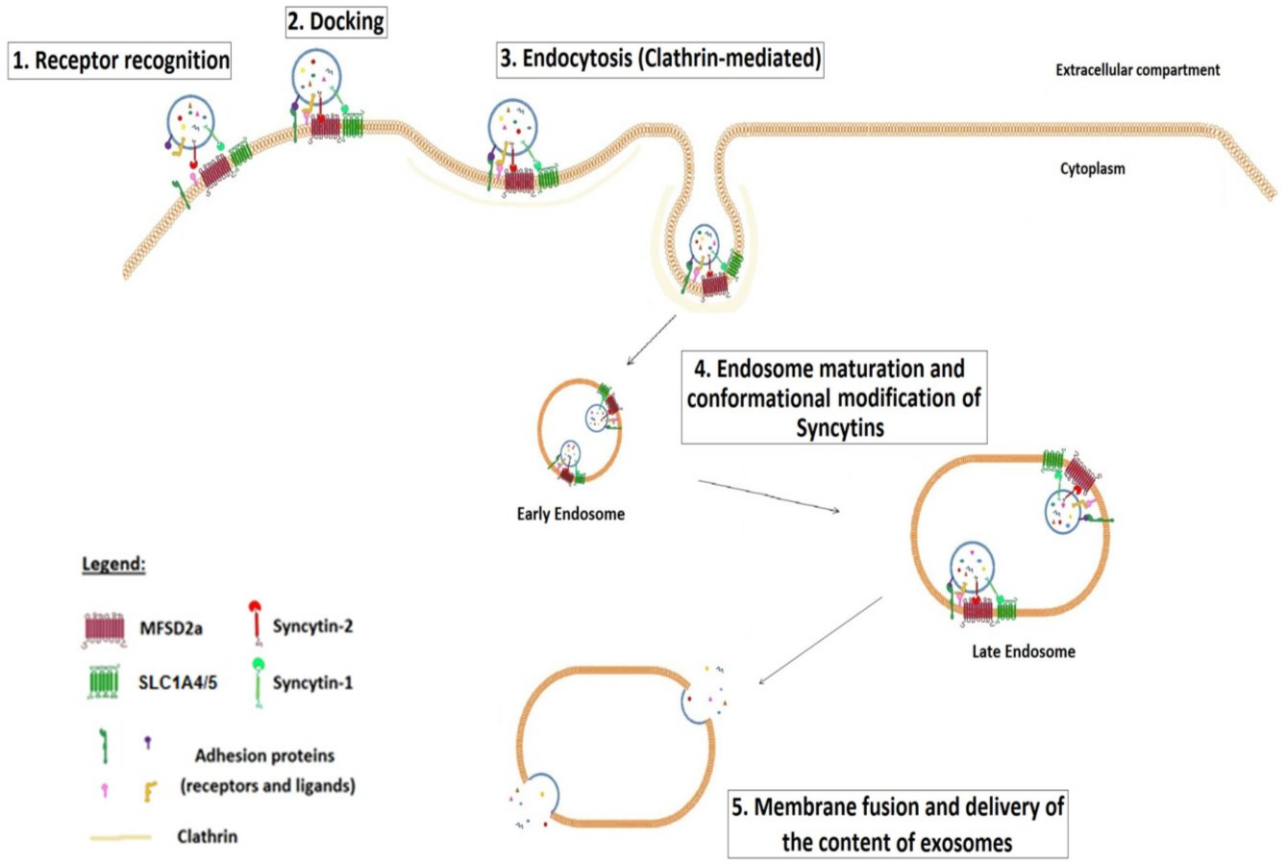

Figure 3. Exosome-associated Syncytin-1 and -2 allow specific entry of exosomes into target cells. Exosomes that harbor Syncytin-1 and -2 specifically target SCL1A4/5- and/or MFSD2a-expressing cells. The recognition and binding of Syncytins to their specific receptor, probably in association with other adhesion proteins (Steps 1 and 2), induce exosome endocytosis (Step 3), being potentially clathrin-mediated. Then, endosome maturation (from early to late endosomes) might bring a conformational change of Syncytins, thereby provoking fusion between exosomal and endosomal membranes (4 and 5). The exosome content is subsequently released in the cytoplasm of target cells, leading to cellular alteration.

5. Placenta Exosomes, Syncytin and Modulation of the Immune Response

The placental exosome can also interact with different immune cell populations and alter their function and activation state [82,83]. One of the early studies had demonstrated that placental exosomes modulated T-cell signaling and that the extent of repression, measured by CD3ζ- and Jak3-reduced expression, correlated with the abundance of FasL at the surface of exosomes [113,114]. Other studies have also revealed that incorporated FasL and TRAIL are activated at the surface of placental exosomes and induce apoptosis of T-cell lines and activated peripheral blood mononuclear cells (PBMC) [115]. Other examples demonstrating an effect of placental exosomes on immune response include the reduction of the cytotoxic function of NK and $CD8^+$ T-cells mediated by incorporated NKG2D ligands and the impact on monocyte recruitment, macrophage differentiation and cytokine production [96,116–118]. As highlighted above, we have demonstrated that Syncytin-1 and -2 are incorporated on the surface of placental exosomes and could actively act in a local or distal environment on the immune response [77,84]. In fact, Holder *et al.* [78] have also showed that placental exosomes alter PBMC activation, presumably through associated Syncytin-1 [119]. Hence, in a similar manner to the exosome-cell interactions described above, placental exosomes could also interact with various immune cell types and, through Syncytin-1 and -2 ISD, be involved in the immunosuppressive state, leading to immunotolerance. It will be important to further understand how Syncytin-1 and -2 incorporated in trophoblast-derived exosomes may mediate such immunosuppression, *i.e.*, whether NK and $CD8^+$ T-cells are being targeted, as suggested for the immunosuppression mediated by the Friend-MuLV envelope protein [71]. In addition, a more comprehensive mechanism of action of ISD on immune cell activation with respect to the exact nature of the cell membrane protein interacting with this domain would be valuable information.

6. Association between Downregulation of Syncytin-1 and -2 and Preeclampsia and Their Use as New Potential Biomarkers

Pre-eclampsia (PE) is a pregnancy disorder associated with a defect in placentation [120,121]. PE is one of the leading causes of maternal and neonatal mortality and morbidity. As appropriate management tools and preventives therapies are needed to achieve proper clinical follow-up of predisposed pregnant women, major efforts are ongoing to identify biomarkers for the early diagnosis of PE. We and others have reported that the expression of Syncytin-1 and -2 is reduced in the placental tissue of PE when compared to the tissue of normal pregnant women [65,122–130]. As PE has been associated with abnormal placentation (*i.e.*, through the reduced size of the syncytiotrophoblast layer) and with an exacerbated inflammatory response, reduced Syncytin-1 and -2 levels might be determinant in these PE-associated features. Furthermore, based on our recent findings that Syncytin-2 is also reduced in serum-derived exosomes from PE patients [84], we are suggesting that exosome-associated Syncytin-2 levels modify their ability to communicate with neighboring or more distant cells, such as endothelial cells, cytotrophoblasts and various immune cell population. This would, in turn, potentially impact normal placental development function and could have detrimental effects on fetomaternal immunotolerance. As a final point, reduced Syncytin-2 levels on the surface of serum-derived PE exosomes from second and third trimester samples warrants further investigation to determine if these exosomes harvested at an earlier time point (first trimester) could be an important

biological material to monitor pregnant women for predisposition toward PE through associated Syncytin-2 protein levels.

7. Conclusions

ERV envelope proteins Syncytin-1 and -2 have been clearly shown to be implicated in normal placenta function through their fusogenic ability to drive cytotrophoblast fusion to the syncytiotrophoblast layer. Through their active immunosuppression domain, these proteins might also modulate the immune response, which otherwise would harm the fetus. Recent findings have now shed light on how these proteins could also act more distally from the placenta through their incorporation in placental exosomes. Incorporated Syncytin proteins could also affect other exosomal functions and be implicated in intercellular communication with other cell types. ERV Syncytin-1 and -2 are also importantly reduced in PE patients, and their implication at the cellular and exosomal levels could have an important consequence in relation to exosomal function related to induced immunosuppression and other placental functions. In addition, the association between Syncytin proteins and placental exosomes opens up the possible use of these proteins as markers of various obstetric disorders, such as pre-eclampsia. In conclusion, more studies are needed to mechanistically address the exact function of these intriguing proteins in placental function, and exciting developments in this area of placenta research are expected to emerge in the upcoming years.

Acknowledgments

This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (#298527-2013) and by an operating grant from the Canadian Institutes of Health Research (CIHR # MOP130561) and from the March of Dimes Foundation (#6-FY13-155) (B.B.). B.B. holds a Canada Research Chair in Human Retrovirology (Tier 2).

Conflicts of Interest

The authors declare no conflict of interest.

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